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ORIGINAL ARTICLE

The immunogenicity of seasonal and pandemic influenza vaccination in autoimmune inflammatory rheumatic patients—a 6-month follow-up prospective study

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Abstract

Introduction Influenza may cause severe complications in patients with autoimmune inflammatory rheumatic disease (AIRD), to whom vaccinations are especially recommended. However, AIRD patients require cautious scrutiny of immunogenicity as they might exhibit poor antibody response to vaccination, especially when taking immunomodulatory medications.

Aim The aim was to determine immunogenicity of seasonal and pandemic influenza vaccine in AIRD patients, its timeline/persistence, and influence of medications on immune response.

Methods One hundred and thirty-seven AIRD and 54 healthy controls were vaccinated with trivalent seasonal influenza. After 3–5 weeks, 15 healthy controls and 93 AIRD were vaccinated with pandemic influenza vaccine, and 63 of patients were vaccinated a second time after 3–5 weeks. Sera were collected before vaccination, 18–90 days after each vaccination, and more than 180 days after the last vaccination. The immune response was measured using hemagglutination inhibition (HI) assay and IgG/IgA antibodies against influenza A/B with ELISA.

Results Our findings indicate that following vaccination with seasonal influenza vaccine, seroprotection, seroresponse, and change in geometric mean titers (GMT) in AIRD patients was not compromised compared to healthy. Similarly, we report for pandemic influenza vaccination little added benefit of the second dose. We confirm lowest increase in HI titer in rituximab-treated AIRD compared to other medications. Vaccination largely tilts the balance from negative ELISA A IgG and IgA titers to positive titers in seasonal H1N1 seroresponsive AIRD patients and controls. A significant decrease in HI GMT and seroprotection was observed only in AIRD at >180 days after vaccination highlighting an absent persistence of immunogenic response in AIRD patients. Due to high initial HI titers for influenza vaccine, we foresee their benefit in personalized medicine in the future.

Conclusion Influenza vaccination is immunologically active for AIRD, with little value of the second dose of the pandemic vaccine and further scrutiny on persistence of immune response to vaccine in AIRD is needed.

Keywords Autoimmune inflammatory rheumatic disease · Influenza · Pandemic · Seasonal · Vaccination

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Background

Influenza is a frequent infectious disease affecting up to 20% of the population yearly [1]. Influenza virus successfully evades host immunity acquired by previous vaccination and infections due to rapid changes in surface antigens [2]. Some populations like the elderly, patients with chronic illnesses, and pregnant women are at high risk for complications of the infection. In Slovenia, vaccination is recommended for the whole population and especially for those older than 65 years, children between the age of 6 months and 2 years, and people with clinical risk, including healthcare workers [3]. In autoimmune inflammatory rheumatic disease (AIRD) patients, the incidence of influenza is higher than in the control population due to a compromised immune system, which is further impaired by medication [4, 5]. In patients with chronic autoimmune diseases, influenza can cause severe complications and represents a major cause of morbidity and mortality [6]. Both disease and immunosuppressive therapy contribute to increased mortality associated with infections. Annual influenza vaccination is recommended by EULAR as a way to prevent infection and its complications in patients with autoimmune rheumatic diseases [7], and the clinician recommendation exerts the most positive influence on patients' decisions to vaccinate [8].

Immunogenicity of a vaccine is the ability of vaccine content particles (antigens) to induce protective immune response against the pathogen. The vaccine composition changes yearly to match prevailing circulating strains and induces a protective antibody response. Critical antibodies are directed against the hemagglutinin molecule to interfere with viral entry. Influenza vaccine as recommended by WHO for 2009/2010 season included inactivated strains of influenza A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007(H3N2), and B/Brisbane/60/2008 [9, 10]. In Slovenia, the vaccine produced by Sanofi Pasteur was used. Additionally, during 2009–2010, because of the outbreak of pandemic influenza virus, A/H1N1pdm09 vaccination was recommended against this virus, due to severe complications observed previously with this infection [11]. The pandemic vaccine containing fragments of A/California/7/2009 (H1N1pdm)-like strain (X-179A) (Glaxo Smith Kline) was offered in Slovenia [9], and immunization was highly recommended for AIRD patients [7].

Very little is known about the (a) protective antibody levels against influenza viruses, (b) the influence of DMARD treatment to vaccine response, and (c) the autoimmune response after multiple vaccinations [12]. An adequately functioning immune system is mandatory for protective immunity after vaccination but AIRD patients, however, exhibit an impaired immune mechanism. We conducted a prospective monocenter, open-label study with a 6-month follow-up. We previously reported on the autoimmune response following influenza vaccination in AIRD patients [9]. The aim of the current study is to determine immunogenicity (seroconversion, seroresponse and seroprotection) of seasonal and pandemic influenza vaccines in AIRD patients and to establish whether treatment influences response to vaccine. The study design allowed longitudinal evaluation of the immune response, which was rarely tracked. Furthermore, we evaluated the persistence of response to vaccine and immunogenicity of the second dose of the pandemic vaccine.

Methods

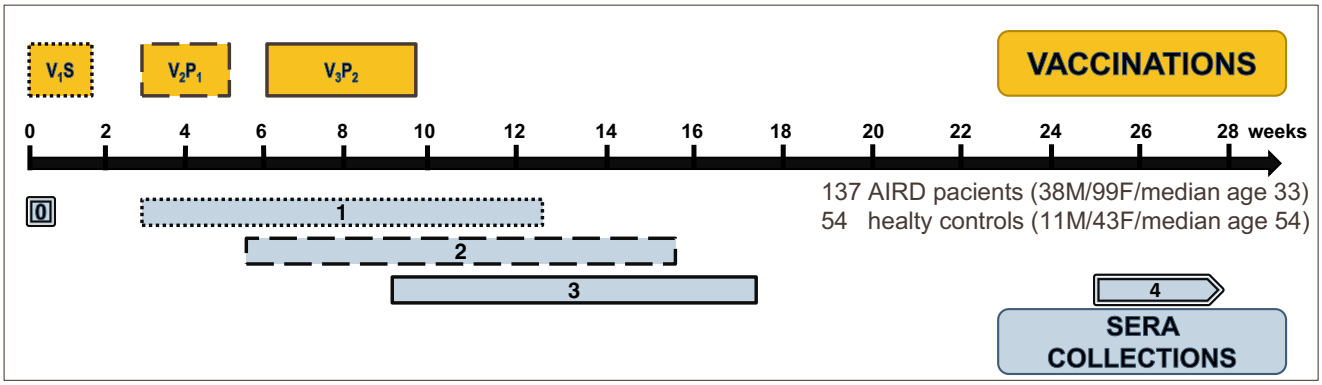
Patients

The following AIRD patients were vaccinated: 109 rheumatoid arthritis, 10 psoriatic arthritis, 15 ankylosing spondylitis, 1 mixed connective tissue disease, 1 juvenile rheumatoid arthritis, and 1 adult Still disease patient. Altogether, 137 patients and 54 apparently healthy controls (health care professionals and medical students) were vaccinated with seasonal vaccine. After 3–5 weeks, 15 healthy controls and 93 patients were vaccinated with pandemic influenza vaccine, and 63 of these patients were vaccinated a second time 3–5 weeks after the first dose of pandemic vaccine (Scheme 1). Vaccination of immunocompromised patients with two doses of pandemic vaccine was recommended by the National Institute of Public Health of Slovenia. Also, 72 AIRD patients comprising of 67 rheumatoid arthritis, 4 ankylosing spondylitis, and 1 dermato-myositis patient refused vaccination and were considered as non-vaccinated AIRD controls. In addition, 18 not-vaccinated healthy controls were asked to participate in the study.

Ethical approval for the study was obtained from the National Medical Ethics Committee of Slovenia (#122/09/09), and the study was conducted according to the principles outlined in the Declaration of Helsinki. All participants signed an informed consent. All participants were followed up for 6 months. Blood samples were collected at inclusion (before the first vaccination) in October 2009. Further, sera were collected 1 month post-vaccination and before application of pandemic vaccine, 1 month after pandemic vaccine administration, and for AIRD patients that were vaccinated twice with the pandemic vaccine, one more serum was collected a month after second vaccination. The final sera were withdrawn in the post-influenza season in May through July 2010, which was more than 6 months post-vaccinations. Non-vaccinated participants' blood samples were collected at the time of inclusion in the study. Sera were stored at − 80 °C until tested.

Vaccine

Vaccination was performed with the trivalent seasonal influenza vaccine (Sanofi Pasteur, USA) [13] containing purified hemagglutinin of A/Brisbane/59/2007 (H1N1), A/Brisbane/10//2007 (H3N2), B/Brisbane/60/2008 (B), and with the



Scheme 1 Study flow chart -legend: V_1S - seasonal influenza vaccination; V_2P_1 - first pandemic influenza vaccination; V_3P_2 - second pandemic influenza vaccination

pandemic influenza vaccine (GlaxoSmithKline, UK) [14] containing A/California/7/2009 (H1N1pdm) strain.

Hemagglutination inhibition (HI) assay

The immunogenicity of vaccines was evaluated with HI assay at National Influenza Center (NIC) at National Laboratory for Health, Environment and Food, Slovenia according to WHO standard procedure [15–17] using human erythrocytes and hemagglutinin antigens represented in both vaccines. Briefly, sera were treated with receptor destroying enzyme (RDE (II), Denka Seiken Co.Ltd) by diluting one volume of serum in four volumes of RDE, incubated overnight and heat inactivated for 30 min at 56 °C. Subsequently, sera were diluted 1:10 with PBS. Serial 2-fold dilutions up to 1:2560 of RDE-treated sera were prepared in a U bottom 96-well microtiter plates and tested against live Madin–Darby canine kidney cells (MDCK) - grown vaccine viruses in the final concentration of 4 HAU/25 μl. Twenty-five microliters of standard virus was added to each diluted test serum and incubated. Then, 0.75% dilution of human red blood cells in PBS was added, and the plates were incubated at 4 °C for 1 h. Plates were read for the highest serum dilution able to inhibit hemagglutination. The reciprocal values of this serum dilution represent the HI titer of the tested serum.

HI titer lower than 40 was defined as negative, higher than 40 as positive, and 40 as borderline. HI titers were determined up to a titer of 2560. Although there is debate about the best correlation of protection against influenza, seroprotection was defined as a HI titer ≥ 40, since this correlation is used by the US Food and Drug Administration [18]. Additionally, seroprotection as HI titer 1:40, was previously suggested to represent a reasonable statistical correlate for efficacy of 50–70% against clinical symptoms of infection based on challenge studies in healthy adults [19].

Seroprotection was defined as HI antibody titers of ≥ 40, seroconversion as postvaccination HI antibody titers ≥ 40 in persons whose prevaccination titers were < 10, and seroresponse as seroconversion or an increase in HI antibody titers ≥ 4-fold in persons, whose prevaccination titers were ≥ 10 [20].

For the purpose of clarity we will use the term persistence of seroresponse to indicate persistence of 4-fold increased HI antibody titers for samples with prevaccination titers ≥ 10 or titers ≥ 40 in patients whose prevaccination titers were < 10 over time.

ELISA

IgG and IgA antibodies against influenza A and B were detected with VIRION SERION ELISA classic Influenza A/B Virus (Institut Virion/Serion GmbH, Würzburg, Germany) following manufacturers' instructions. Antigens used in this test are conserved nucleoproteins and matrix proteins. An ELISA antibody titer lower than 10 E/ml is defined as negative, while a titer of 10–15 E/ml as borderline and titer > 15 E/ml as positive.

Statistical analysis

Geometric mean titers (GMT) of autoantibodies and 95% confidence interval (CI) were calculated to assess the immunogenicity of the whole group. To calculate statistics, GraphPad Prism 5 was used. Non-parametric tests were used due to non-normal data distribution (Mann–Whitney or Kruskal–Wallis, followed by Dunn multiple comparison) to compare GMT between groups and for paired data (the same group before/after vaccination) Wilcoxon matched pair signed ranked test. For categorical data Fischer exact test was used.

Results

Baseline population characteristics regarding immunity against seasonal and pandemic influenza

Vaccinated, non-vaccinated patients and control median (min–max) age were 54 (19–79), 56 (45–67), 33 (21–62) and 32

Table 1 Response to seasonal influenza vaccination in AIRD and controls

	Controls (V-C)	AIRD (V-AIRD)	Mann–Whitney test/ Fischer exacts test	Wilcoxon pair signed rank test
Number (M/F)	54 (M 11/ F 43)	137 (M 38/F 99)		
Age median (min–max)	33 (21–62)	54 (19–79)		
ELISA A IgG				
Before			Before	
GMT (95% CI)	10.2 (8.1–12.7)	6.5 (5.6–7.8)	p** between V-C and V-AIRD	
Neg/borderline/pos	27/10/17	87/19/31		
After			After	Before–after for
GMT (95% CI)	17.7 (14.5–21.8)	13.7 (11.9–15.8)	p* between V-C and V-AIRD	V-C: p****
Neg/borderline/pos	9/17/28	52/25/60		V-AIRD: p****
ELISA A IgA				
Before			Before	
GMT (95% CI)	5.5 (4.2–7.2)	3.9 (3.4–4.6)	p* between V-C and V-AIRD	
Neg/borderline/pos	38/6/10	119/6/12		
After				Before–after for
GMT (95% CI)	9.7 (7.5–12.7)	13.0 (10.7–15.8)		V-C: ****
Neg/borderline/pos	26/8/20	61/10/66		V-AIRD: ****
ELISA B IgG				
Before			Before	
GMT (95% CI)	11.6 (9.0–14.9)	6.8 (5.7–8.2)	p*** between V-C and V-AIRD	
Neg/borderline/pos	23/12/19	92/12/23		
After			After	Before–after for
GMT (95% CI)	17.5 (13.7–22.5)	10.4 (8.9–12.2)	p*** between V-C and V-AIRD	V-C: p****
Neg/borderline/pos	15/11/28	74/19/44		V-AIRD: p***
ELISA B IgA				
Before			Before	
GMT (95% CI)	5.6 (4.3–7.2)	4.3 (3.6–5.1)	p* between V-C and V-AIRD	
Neg/borderline/pos	38/5/11	106/12/19		
After				Before–after for
GMT (95% CI)	8.7 (6.5–11.7)	11.0 (9.1–13.3)		V-C: ****
Neg/borderline/pos	30/6/18	66/20/51		V-AIRD: ****
HI H1N1				
GMT (95% CI) before vaccination	64.2 (50.5–81.6)	45.8 (36.2–57.9)		Before–after for
GMT (95% CI) after vaccination	133.9 (100.8–177.8)	182.6 (141.0–236.5)		V-C: ****
				V-AIRD: ****
Seroprotection before vaccination	74% (40/54)	58% (79/137)	V-C vs V-AIRD *	
Neg/borderline/pos	14/9/31	58/11/68		
Seroprotection after vaccination	92% (50/54)	89% (122/137)		
Neg/borderline/pos	4/8/42	15/14/108		
Seroresponse after vaccination	24% (13/54)	50% (67/133)	V-C vs V-AIRD **	
HI H3N2				
GMT (95% CI) before vaccination	709.6 (480.2–1048.0)	131.3 (114.4–150.7)	p**** between V-C and V-AIRD	
GMT (95% CI) after vaccination	1065.0 (780.5–1454)	312.0 (256.4–381.0)	p**** between V-C and V-AIRD	Before–after
				V-C: p*
				V-AIRD: ****
Seroprotection before vaccination	100% (54/54)	97% (133/137)		
Neg/borderline/pos	0/5/49	4/10/123		
Seroprotection after vaccination	100% (54/54)	100% (54/54)		
Neg/borderline/pos	0/0/54	0/4/133		

Table 1 (continued)

	Controls (V-C)	AIRD (V-AIRD)	Mann–Whitney test/ Fischer exacts test	Wilcoxon pair signed rank test
Seroresponse after vaccination	47% (8/17)	28% (38/134)		
HI B				
GMT (95% CI) before vaccination	131.2 (91.3–188.5)	116.1 (96.7–139.5)		Before–after for
GMT (95% CI) after vaccination	253.8 (190.7–337.8)	262.6 (220.5–312.6)		V-C: p***
				V-AIRD: p****
Seroprotection before vaccination	83% (45/54)	90% (123/137)		
Neg/borderline/pos	9/1/44	14/15/108		
Seroprotection after vaccination	100% (54/54)	98% (134/137)		
Neg/borderline/pos	0/2/52	3/2/132		
Seroresponse after vaccination	34% (17/50)	33% (43/129)		

Baseline serum sample withdrawn at vaccination time (-3 to +5 days), next sample 18-90 days after vaccination (median (IQR) for V-C 31 (29-34) days after vaccination, V-AIRD 31 (22-37) days after vaccination); Seroconversion was calculated only for those who had baseline titers <10. Due to high baseline HI titers four fold increase (seroresponse) was not measurable in 4 V-AIRD for H1N1, 37 V-C and 3 V-AIRD for HI H3N2 and 4 V-C and 8 V-AIRD for HI B. Fisher exact test was used for seroprotection analyses. In V-C 11 were also vacciantedwith pandemic vaccine 1 dose, in V-AIRD 36 were vaccinated only with seasonal, 15 sesasonal+pandemic 1 dose and 86 seasonal+pandemic 2 doses.

GMT geometric mean titer, *IQR* interquartile range, *95% CI* 95% confidence interval, *HI* hemagglutination inhibition

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

(25–38) respectively, and all groups consisted of 18–27% male (Supplemental Table I). Patients used methotrexate, sulfasalazine, leflunomide, chloroquine, adalimumab, etanercept, rituximab, tocilizumab, infliximab, and methylprednisolone and combinations of drugs for therapy. HI showed rather high seroprotection before vaccination with seasonal influenza vaccine (≥ 90%) for H3N2 and B influenza antigens in AIRD who were later vaccinated or not, while in control group (later vaccinated and non-vaccinated) seroprotection for these two antigens were 72–100% (Supplemental Table I). Seroprotection for the seasonal H1N1 influenza antigen in non-vaccinated AIRD patients and controls was observed in higher percentages than in vaccinated AIRD and controls (92%, 83% vs 58%, 74% respectively). Baseline seroprotection against the new, pandemic influenza H1N1pdm was observed in 25–33% of cases, except for healthy controls who were later vaccinated, where it was 81% (Supplemental Table I). The baseline sera of AIRD patients and controls, who were later vaccinated compared to non-vaccinated contained increased levels of IgG and IgA antibodies against Influenza nuclear protein A and B as measured by ELISA. Specifically, in controls, IgG titer ≥ 15 (positive or borderline) was present in 50% (ELISA A) and 57% (ELISA B) of vaccinated controls and only 5% (ELISA A) and 16% (ELISA B) of non-vaccinated controls, while in vaccinated AIRD patients, the percentages were 36%, 27% and non-vaccinated AIRD 8%, 12% respectively.

Response to seasonal influenza vaccine

GMT, seroresponse, seroconversion, and seroprotection were calculated for samples collected 21–42 days (3–6 weeks) and 43–90 days after vaccination. The two groups were united due to similarity of results as response after 18–90 days (Table 1). Eighteen to 90 days after vaccination HI titers for seasonal H1N1, H3N2, and B significantly increased in healthy controls and AIRD patients (Table 1). Seroprotection was achieved in 89–100% of controls and AIRD patients, and both groups developed significantly increased HI GMT after vaccination. There was no difference between AIRD and controls in HI GMT after vaccination with seasonal H1N1 and B, but AIRD patients did have significantly lower HI GMT for H3N2 than healthy controls (Table 1).

Interestingly, analyzing seroresponse to influenza vaccine we noticed, that only a few AIRD (12%) and controls (6%) who could respond to all three antigens actually developed a 4-fold increased antibody titers or seroconversion to all three antigens, while the majority developed seroresponse to only two antigens (Fig. 1).

Analysis of the influence of immunosuppressive drugs on vaccination showed high levels of seroprotection against H3N2 and B even before vaccination in all medications, with

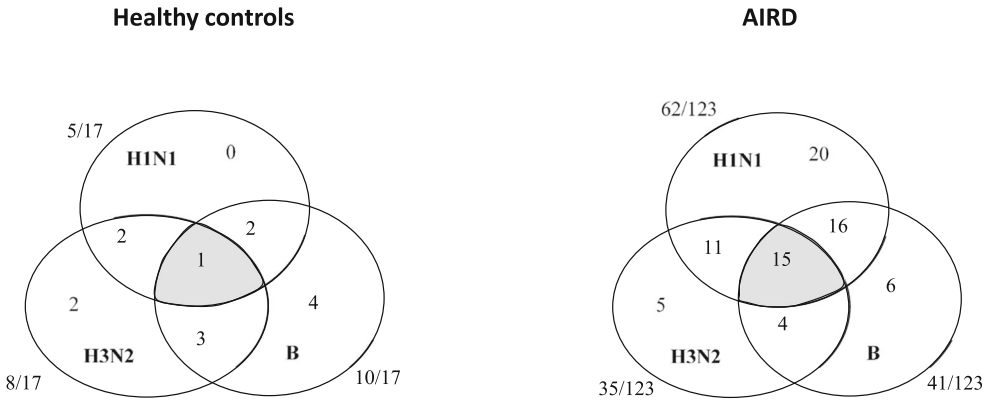


Fig. 1 Seroresponse to antigens in seasonal influenza vaccine. Legend: Patients ($n = 123$) and controls ($n = 17$) who could develop seroresponse to all antigens H1N1, H3N2, and B were analyzed and numbers of those who did serorespond are written in appropriate field

only exception of rituximab and methotrexate treated patients who were not 100% seroprotected for B after vaccination (89% and 96%). For seasonal H1N1, we could observe poorest seroprotection (56%) in patients having rituximab therapy, while methotrexate, adalimumab, etanercept, and tocilizumab treated patients were seroprotecected in 86–91% and vaccinated controls 92%. Patients receiving other drugs reached seroprotection in 100% for seasonal H1N1 after vaccination (Fig. 2). Majority of treated patients responded with at least slight increase (> 1.1-fold) in HI titer for all three antigens from vaccine, while at least 50% rituximab treated patients did not developed any increase in HI titers (Fig. 3). The percentages of those, with increased HI titer (> 1.1-fold) among treated AIRD patients, are higher than non-treated patients for all medications used, except rituximab (for seasonal H1N1, H3N2, B: non-treated 50%, 25%, 63%; rituximab treated 44%, 22%, 50% and vaccinated controls 65%, 54%, 58%, respectively) (Table 2). Patients received rituximab median 85 days before vaccination (range 228–0) and 108 days (range 73–730) after vaccination. Only two of nine rituximab treated patients developed more than 4-fold increase in HI titer to at least one antigen after receiving seasonal vaccine—those two patients received rituximab 102 and 85 days before and 73 and 98 days after vaccination.

As we measured HI titers, as well as nucleoprotein/matrix protein antibody titers before and after vaccination for patients and controls, we discovered that those who responded with a 4-fold increase of HI titer for H1N1 also raised titers of antibodies against nuclear proteins of influenza A and B from negative to positive in 31–70% of cases while those who did not respond with change in HI titer this shift from negative to positive ELISA titers occured in 0–20% (Table 3).

For longitudinal analysis, the samples from 24 healthy vaccinated controls and 109 AIRD patients were analyzed

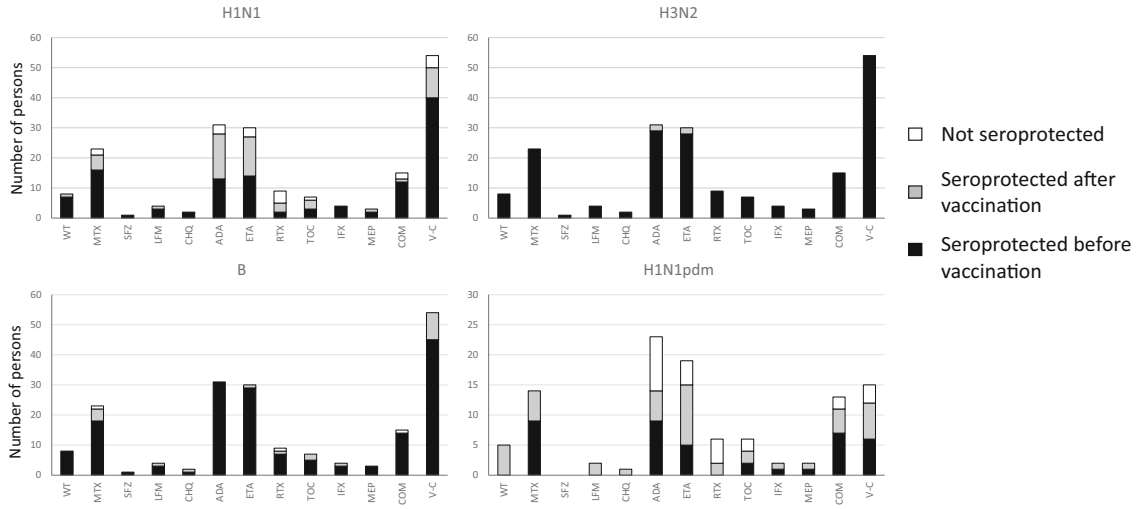


Fig. 2 Levels of seroprotection after seasonal and pandemic influenza vaccine. Legend: High levels of seroprotection were reached after seasonal influenza vaccine for all three strains as well as for H1N1pdm after first vaccination with pandemic vaccine in AIRD on different drugs. Patients treated with MTX did not reach 100% seroprotection for two antigens and rituximab treated patients developed lowest percent of seroprotection after seasonal influenza vaccination, while after 1st pandemic vaccination patients on few biologicals were not 100% seroprotected. MTX methotrexate, SFZ sulfasalazine, LFM leflunomide, CHQ chloroquine, ADA adalimumab, ETA etanercept, RTX rituximab, TOC tocilizumab, IFX infliximab, MEP methylprednisolone, COM combinations of drugs, V-C vaccinated controls, WT without treatment

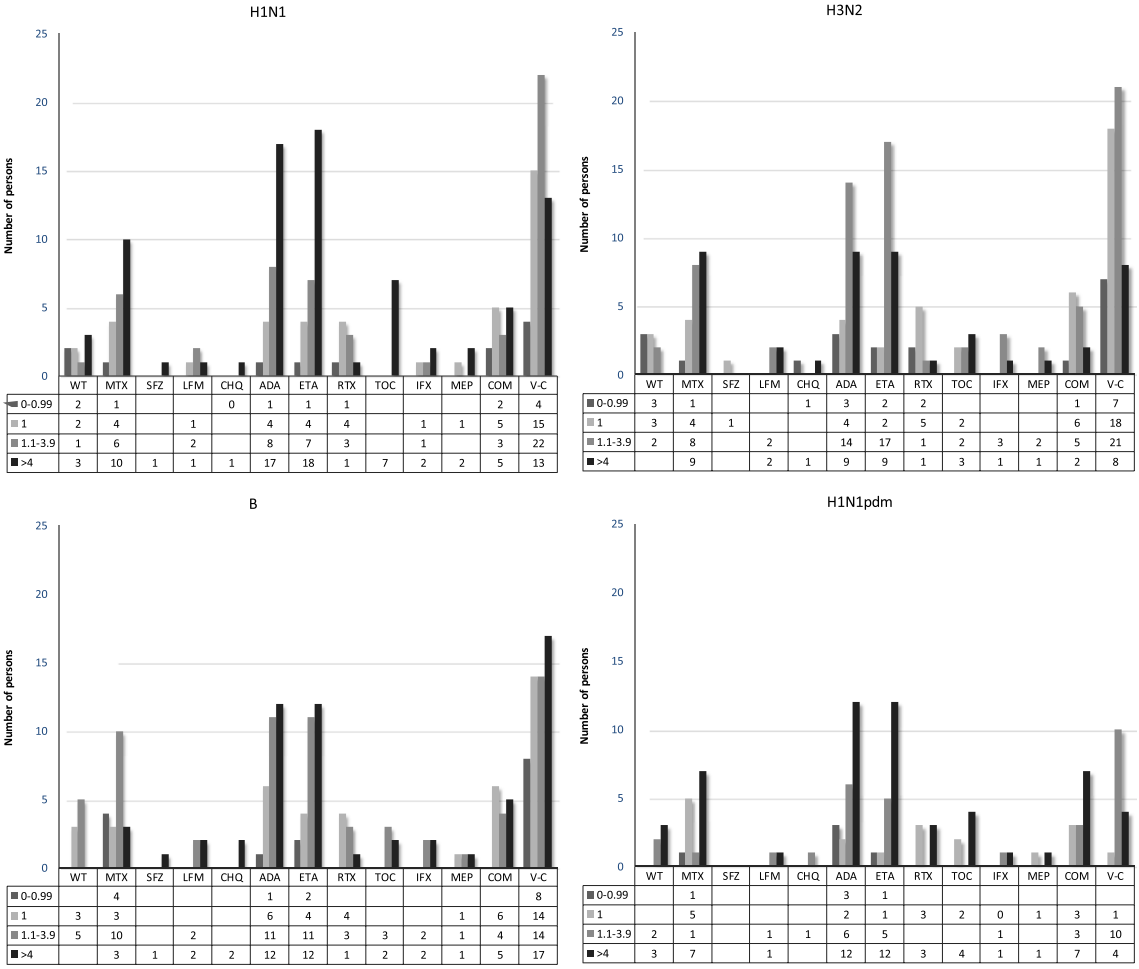


Fig. 3 Number of subjects with fold increase in HI titer after influenza vaccination. Legend: Changes in HI titer for H1N1, H3N2, and B 18–90 days after seasonal influenza vaccination and H1N1pdm HI titer 18–42 days after 1st pandemic vaccination. Majority of patients using drugs developed increased HI titers (1.1-fold increase or more—last two columns in each drug are highest), except for rituximab treated patients, where majority did not change HI titer. Those whom we could not measure > 4-fold increase of titer due to high baseline titer were not included in analysis. MTX methotrexate, SFZ- sulfasalazine, LFM leflunomide, CHQ chloroquine, ADA adalimumab, ETA etanercept, RTX rituximab, TOC tocilizumab, IFX infliximab, MEP methylprednisolone, COM combinations of drugs, V-C vaccinated controls, WT without treatment

Table 2 Change of HI titer after seasonal influenza vaccination

Treatment:	H1N1			H3N2			B		
	No. of patients with increased HI titer (> 1.1)	No. of all treated with drug	% Patients with increased HI titer (> 1.1)	No. of patients with increased HI titer (> 1.1)	No. of all treated with drug	% Patients with increased HI titer (> 1.1)	No. of patients with increased HI titer (> 1.1)	No. of all treated with drug	% Patients with increased HI titer (> 1.1)
None	4	8	50%	2	8	25%	5	8	63%
Methotrexate	16	21	76%	17	22	77%	13	20	65%
Sulfasalazine	1	1	100%	0	1	0%	1	1	100%
Leflunomide	3	4	75%	4	4	100%	4	4	100%
Chloroquine	1	1	100%	1	2	50%	2	2	100%
Adalimumab	25	30	83%	23	30	77%	23	30	77%
Etanercept	25	30	83%	26	30	87%	23	29	79%
Rituximab	4	9	*44%*	2	9	*22%*	4	8	*50%*
Tocilizumab	7	7	100%	5	7	71%	5	5	100%
Infliximab	3	4	75%	4	4	100%	4	4	100%
Methylprednisolone	2	3	67%	3	3	100%	2	3	67%
Combinations	8	15	53%	7	14	50%	9	15	60%
Controls (V-C)	35	54	65%	29	54	54%	31	53	58%

Table 3 Response to antigens not included in the vaccine after influenza vaccination

		V-C		V-AIRD		V-C		V-AIRD		V-C		V-AIRD	
		#neg/pos*	%	#neg/pos*	%	#neg/pos*	%	#neg/pos*	%	#neg/pos*	%	#neg/pos*	%
Seasonal		HI titer to H1N1 change > 4-fold				HI titer H1N1 change 1.1–3.9-fold				HI titer H1N1 change ≤ 1-fold			
ELISA A	IgG	7/10	70	20/48	42	1/10	10	3/19	16	1/7	14	0/13	0
	IgA	4/12	33	36/62	58	0/13	0	11/28	39	4/20	20	3/29	10
ELISA B	IgG	3/7	43	16/48	33	1/7	14	1/20	5	0/9	0	0/12	0
	IgA	4/10	40	16/51	31	0/16	0	6/26	23	3/24	12	4/29	14
Pandemic		HI titer to H1N1pdm change > 4-fold				HI titer H1N1pdm change 1.1–3.9-fold				HI titer H1N1pdm change ≤ 1-fold			
ELISA A	IgG	1/4	25	7/23	30	0/4	0	2/6	33	0/2	0	3/11	27
	IgA	0/3	0	11/28	39	0/6	0	0/7	0	0/1	0	0/12	0
ELISA B	IgG	0/2	0	4/25	16	1/3	33	1/10	10	0/1	0	2/13	15
	IgA	0/6	0	2/30	7	0/4	0	0/10	0	0/1	0	0/11	0

Those who developed > 4-fold increased HI titers to seasonal H1N1 after vaccination also in large proportion changed conserved nucleoprotein and matrix proteins IgG and IgA antibody titers from negative (< 10) to positive (> 15).

V-C vaccinated controls; *V-AIRD* vaccinated autoimmune inflammatory rheumatic disease patients

*Number of ELISA negative before vaccination, becoming positive after vaccination / number of ELISA negative before vaccination

(Table 4). Increased HI GMT after 18–90 days were significantly lower in AIRD at > 180 days, while the difference was not significant in controls. Also, seroprotection provoked at 18–90 days in AIRD was significantly diminished > 180 days after vaccination for seasonal H1N1 but not in controls (against influenza H3N2 and B there were 89% or more cases seroprotected already at baseline, so it is hard to conclude on rise and persistence). Similarly, seroresponse seen at 18–90 days was significantly diminished in > 180 days in AIRD but not in controls for all three seasonal influenza antigens (Table 4).

We can confirm that the majority of those seroprotected at baseline, stayed seroprotected after 180 days (controls and AIRD 11/11, 48/52 for H1N1; 24/24, 91/97 for H3N2; 20/22, 79/84 for B).

Focusing only on the patients who responded to vaccination, in one third of vaccinated patients who were not seroprotected against seasonal influenza H1N1 and B at baseline, but were seroprotected at 18–90 days, HI antibody titers were below levels of seroprotection 180 days after vaccination (for H1N1 11/30; H3N2 0/2; B 3/10). Achieved seroconversion in the AIRD group against seasonal H1N1 virus was transient during the study and decreased after 180 days (reached in 68% of patients in 18–90 days (11/16), still present in 19% after > 180 days (3/16)), namely, 72% (8/11) of AIRD that reached seroconversion for seasonal H1N1 at days 18–90 no longer showed seroprotection level of antibodies after > 180 days. We cannot compare this with controls or H3N2 and B, as there were no patients who had baseline titers < 10 in longitudinal study group. In AIRD with seroresponse at days 18–90, the level of antibodies did not persist at > 180 days in 62% (37/60), 60% (20/33), and 77% (27/35) for seasonal H1N1, H2N3, and B respectively, while the loss of seroresponse over time in vaccinated healthy controls was lower (20% (1/5), 0% (0/1), 40% (2/5) for seasonal H1N1, H2N3, B, respectively). Because numbers of seroresponsive patients treated with each medication in longitudinal study were not high (especially for B), it is hard to draw conclusions, but the drop of antibody titer was not typically related to any medication used as we observed loss of seroresponse titers for H1N1, H3N2 and B in patients treated with methotrexate in 78% (7/9), 88% (7/8) and 100% (2/2), with adalimumab 70% (12/17), 62% (5/8), and 82% (9/11) and with etanercept 40% (6/15), 43% (3/7), and 90% (9/10), respectively (Fig. 4). We further investigated if vaccination with vaccine against pandemic influenza influences persistence of seroresponse to seasonal influenza vaccine antigens (Fig. 5). Among those patients who had seroresponse for seasonal H1N1, H3N2, and B still present after > 180 days, 34% (8/23), 38% (5/13), and 62% (5/8), respectively were vaccinated only against seasonal influenza. Among those who lost seroresponse, 32% (12/37), 35% (7/20), and 30% (8/27) were vaccinated only against seasonal influenza, meaning that additional vaccinations did not change the persistence of seroresponse.

Response to pandemic influenza vaccine

For pandemic influenza, controls were vaccinated only once, while due to recommendation, the majority of AIRD patients (63/93) received vaccination twice. HI GMT to H1N1pdm increased to a similar level in healthy controls and patients after the first vaccination (from 24 and 19 and to 60 and 90) (Table 5; Fig. 6), as well as seroprotection was reached in similar shares (before vaccination 40%/37% and after

Table 4 Longitudinal analysis of seasonal influenza vaccination response

	Baseline		18–90 days		> 180 days		Wilcoxon-paired signed rank test or Fisher exact test
	V-C	V-AIRD	V-C	V-AIRD	V-C	V-AIRD	
Time (median (IQR))	0 (0–1)	0 (0–1)	32 (31–35)	30 (22–37)	234 (227–242)	218 (208–230)	
HI H1N1							
GMT titer (95% CI)	51.6 (36.7–72.6)	42.0 (32.3–54.7)	124.9 (82.6–188.9)	190.5 (142.6–254.5)	85.8 (60.8–121.1)	88.3 (67.6–115.4)	V-C: Baseline/18 days*** 18–180 days** AIRD: Baseline/18 days*** 18–180 days***
Seroprotection Neg/borderline/pos	63% (15/24) 9/4/11	57% (62/109) 47/10/52	96% (23/24) 1/5/18	90% (98/109) 11/10/88	88% (21/24) 3/3/18	72% (79/109) 30/14/65	V-C Baseline/18 days* AIRD: Baseline/18 days*** 18–180 days**
Seroconversion				68% (11/16)		19% (3/16) 27% (3/11)* (for18-90 days)	
Seroresponse			20% (5/24)	56% (60/107)	17% (4/24)	21% (23/107)	AIRD: 18–180 days***
HI H3N2							
GMT titer (95% CI)	1397 (1130–1726)	121.2 (103.9–141.3)	1868 (1595–2189)	309.5 (246.2–389.0)	1552 (1264–1907)	176.2 (146.5–211.8)	V-C: Baseline/18 days* AIRD: Baseline/18 days*** 18–180 days***
Seroprotection Neg/borderline/pos	100% (24/24) 0/0/24	96% (105/109) 4/8/97	100% (24/24) 0/0/24	100% (109/109) 0/4/105	100% (24/24) 0/0/24	99% (108/109) 1/8/100	
Seroresponse			50% (1/2)	31% (33/106)	50% (1/2)	12% (13/106)	AIRD: 18–180 days**
HI B							
GMT titer (95% CI)	205. (118.3–357.4)	112.9 (91.3–139.6)	340.0 (221.2–522.4)	251.3 (206.1–306.4)	230.8 (140.1–380.1)	145.7 (117.4–180.7)	V-C: 18–180 days** AIRD: Baseline/18 days*** 18–180 days***
Seroprotection Neg/borderline/pos	96% (23/24) 1/1/22	89% (97/109) 12/13/84	100% (24/24) 0/1/23	98% (107/109) 2/1/106	100% (24/24) 0/3/21	94% (103/109) 6/8/95	
Serorseponse			25% (5/20)	35% (35/101)	15% (3/20)	8% (8/101)	AIRD: 18–180 days***

Seroresponse after 180 days is calculated regarding baseline titers. Seroconversion is stated only for H1N1 AIRD as in longitudinal study for H1N1 controls and for H3N2 and B controls and AIRD there was no subjects with baseline titer < 10. Fisher exact test was used for seroprotection, seroconversion, and seroresponse analyses

V-C vaccinated controls, *V-AIRD* vaccinated autoimmune inflammatory rheumatic disease patients, *GMT* geometric mean titer, *95% CI* 95% confidence interval

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ significance

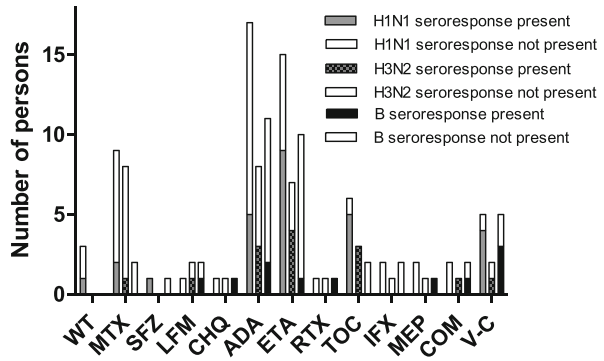


Fig. 4 Does treatment influence persistence of seroresponse > 180 days? Legend: Persistence of seroresponse to seasonal influenza antigens at > 180 days was tested in subjects who had seroresponse at 19–90 days based on drugs used. MTX methotrexate, SFZ sulfasalazine, LFM leflunomide, CHQ chloroquine, ADA adalimumab, ETA etanercept, RTX rituximab, TOC tocilizumab, IFX infliximab, MEP methylprednisolone, COM combinations of drugs, V-C vaccinated controls, WT without treatment

vaccination 80%/77% in controls and AIRD respectively). After the second vaccination, seroprotection was acquired in only additional 7% of patients (79% to 86%) (Table 5). Also, after the first vaccination, seroresponse was seen in 52% of AIRD (as compared to 27% in controls), after the second vaccination in additional 24% of AIRD (Table 5). We observed, similarly to seasonal influenza vaccination, increased titers of IgG, IgA antibodies against nuclear protein of influenza A after first pandemic influenza vaccination of AIRD, but this additional increase was not observed after second vaccination (Table 5).

Investigating persistence of vaccination effect (Table 6), HI GMT of controls and once or twice vaccinated AIRD were similar > 180 days following vaccination, while seroprotection was lower in AIRD vaccinated twice (45% compared to 82% in those vaccinated once). Persisting seroresponse, as compared to baseline, was also similar in controls, once vaccinated and twice vaccinated AIRD (33%, 27%, 28%, respectively).

Discussion

Vaccination against influenza is a strategy to reduce mortality and morbidity associated with influenza in AIRD; however, in order to be a good strategy, vaccination must be safe and effective. The present study was conducted in the 2009–2010 season, when more than 90% of all influenza cases in Europe and Slovenia were caused by a novel pandemic Influenza A H1N1 virus [21]. Two separate types of influenza vaccine (the 2009–2010 seasonal influenza vaccine and the 2009 pandemic H1N1 vaccine) were used and we had the opportunity to evaluate their influence on efficacy and persistence of antibody level for antigens included in the vaccines. The committee for Proprietary Medicinal Products (CPMP) at European Medicinal Agency (EMA) issued guidelines on influenza vaccines, where they suggest immunological testing of vaccines. Their guidelines state that the cutoff of vaccine immunogenicity for the general population are seroprotection > 70%, seroconversion > 40% and a factor of increase GMT > 2.5-fold. To meet immunogenicity, each antigen must meet at least one criterion [22].

Our results confirmed a satisfactory humoral immune response for seasonal influenza H1N1 antigen after vaccination of AIRD and controls. The seroprotection antibody level before vaccination was observed in 74% and 58%, respectively and reached similar percentage in both groups 18–90 days after vaccination (92%, 89% (Table 1)) or in the longitudinal cohort (96%, 90% (Table 4) respectively). Baseline

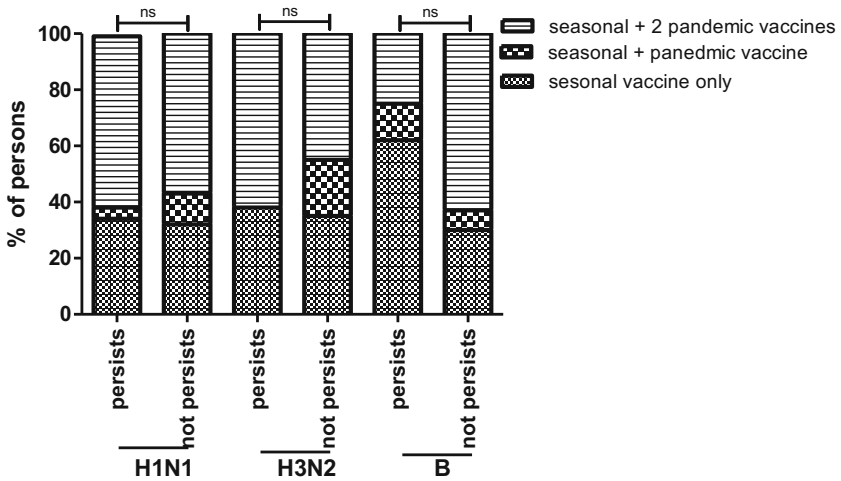


Fig. 5 Influence of pandemic additional vaccinations to persistence of seroresponse to seasonal antigens in AIRD. Legend: Seroresponse to seasonal influenza antigen seen at 18–90 days was not dependant on additional pandemic influenza vaccinations at > 180 days—% of vaccinated with seasonal or seasonal + pandemic was similar in those with persisting and those with not persisting seroresponse at >180 days

Table 5 Response to pandemic influenza vaccination in AIRD patients and controls

	Controls after 1st vaccination	All AIRD after 1st vaccination	AIRD after 2nd vaccination	Wilcoxon-paired signed ranked test comparison before-after/ Fischer exact
Number (M/F)	15 (6/9)	93 (31/62)	63 (24/39)	
Time after vacc median (IQR)	28 (27–31)	21 (21–25)	29 (28–30)	
Age median (min–max)	38 (22–58)	56 (19–82)	55 (34–79)	
ELISA A IgG				
Before				
GMT (95% CI)	9.6 (5.9–15.5)	11.4 (9.3–19.9)	17.9 (14.1–22.7)	Controls *** AIRD 1st **
Neg/borderline/pos	10/2/3	40/18/35	15/11/37	
After				
GMT (95% CI)	15.4 (9.9–23.9)	17.2 (14.3–20.7)	19.7 (16.0–24.3)	
Neg/borderline/pos	5/5/5	22/17/54	14/9/40	
ELISA A IgA				
Before				
GMT (95% CI)	7.2 (3.7–14.0)	10.1 (8.0–12.9)	15.1 (11.4–19.9)	Controls ** AIRD 1st *** AIRD 2nd *
Neg/borderline/pos	10/0/5	48/10/35	24/3/36	
After				
GMT (95% CI)	10.3 (5.6–18.8)	16.3 (12.8–20.7)	13.5 (10.5–17.4)	
Neg/borderline/pos	9/1/5	35/5/53	20/11/32	
ELISA B IgG				
Before				
GMT (95% CI)	13.1 (8.2–20.7)	10.0 (8.1–12.4)	10.2 (8.0–13.0)	
Neg/borderline/pos	6/3/6	47/18/28	31/10/22	
After				
GMT (95% CI)	13.4 (7.9–22.6)	10.6 (8.7–12.9)	10.8 (8.5–13.8)	
Neg/borderline/pos	5/5/5	45/13/35	31/9/23	
ELISA B IgA				
Before				
GMT (95% CI)	5.7 (3.1–10.3)	8.8 (6.8–11.3)	8.6 (6.5–11.3)	AIRD 2nd **
Neg/borderline/pos	9/3/3	59/17/17	37/9/17	
After				
GMT (95% CI)	5.4 (3.0–9.9)	9.0 (7.2–11.4)	7.6 (5.8–9.8)	
Neg/borderline/pos	11/1/3	53/11/29	39/7/17	
HI H1N1pdm				
GMT (95% CI) before vaccination	24.2 (15.7–37.3)	19.5 (16.7–24.2)	86.1 (60.2–123.2)	Controls **
GMT (95% CI) after vaccination	60.9 (40.5–91.5)	90.6 (66.3–123.7)	149.8 (107.6–208.6)	AIRD 1st *** AIRD 2nd ***
Seroprotection before vaccination	40% (6/15)	37% (34/93)	79% (50/63)	Controls vs AIRD 2nd ***
Neg/borderline/pos	9/3/3	59/17/17	13/7/43	
Seroprotection after vaccination	80% (12/15)	77%(72/93)	86% (54/63)	
Neg/borderline/pos	3/4/8	21/10/62	9/2/52	
Seroconversion after vaccination	0/0	68% (13/19)	0% (0/3)	
Seroresponse after vaccination	27% (4/15)	52% (48/93)	24% (14/58)	Controls vs AIRD 1st **

Serum samples were collected at time of pandemic influenza vaccination (− 9 to + 3 days). Next sample 18–42 days after 1st vaccination. Five controls and 4 AIRD were vaccinated only with pandemic vaccine; the rest of included patients were vaccinated with pandemic and seasonal vaccine. For second vaccination, serum was withdrawn (− 9 to + 3 days) prior second vaccination and 18–42 days after second vaccination. Seroconversion/seroresponse after 2nd vaccination calculated based on time at 2nd vaccination and not baseline status. Only 19 patients had baseline titer < 10 before 1st vaccination and 3 patients before 2nd vaccination, so seroconversion is calculated only for these 3. Of 63 patients who were vaccinated with 2nd dose, 5 had titers before 2nd vaccination, so high we could not calculate seroresponse (> 4-fold increase); so seroresponse of 58 were calculated

GMT geometric mean titer, *95% CI* 95% confidence interval, *HI* hemagglutination inhibition

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ significance

seroprotection against influenza H3N2 and B was very high in controls and AIRD (> 83%) and increased to > 95% in both groups. Seroprotection against pandemic influenza H1N1pdm was determined to be 37% in AIRD and 40% in controls and

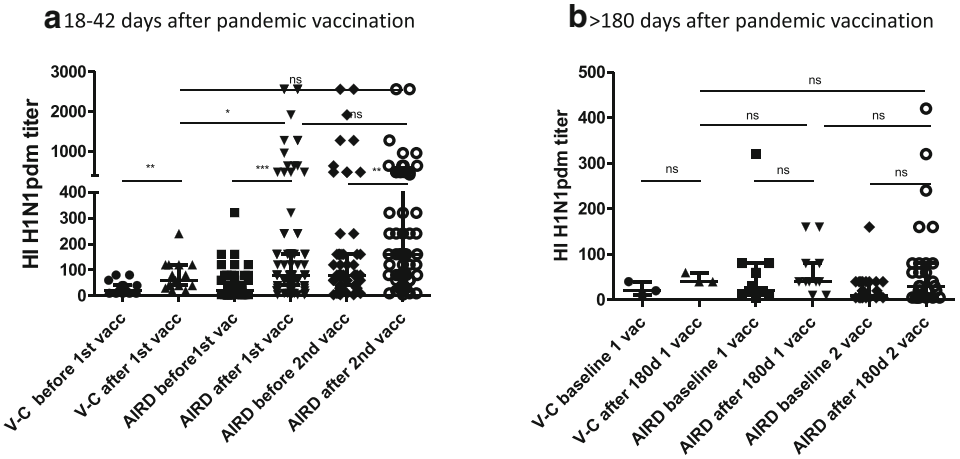


Fig. 6 How does second pandemic vaccination influence HI titers. Legend: Titers of antibodies against pandemic influenza vaccine antigen after vaccination of controls (V-C) and AIRD patients (AIRD). Significance *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

increased to 77% and 80% after vaccination. Due to high percentage of patients with existing protective antibodies against seasonal influenza at inclusion in the study, a low number of serconversions was possible. In the light of this fact, it is hard to comment the first two requirements of guidelines, but from our data, HI GMT increased more than 2.5-fold in AIRD patients for all three antigens in seasonal influenza vaccine (Table 1) as well as for the pandemic influenza H1N1pdm strain (Table 5). This further confirms good immunogenicity of vaccines in AIRD patients which is in concordance with the findings of several previous studies [20, 23–26] showing that influenza vaccines are generally effective for AIRD patients.

Studies have found that on the basis of achievement of seroprotection, the efficacy of seasonal influenza vaccination of healthy individuals and patients with RA, regardless of the concomitant treatment, should be similar [27–29]. Few studies reported a modestly impaired humoral response in those patients treated with rituximab and mixed results when using anti-TNF drugs [23, 24, 30–32]. Vaccine protection was also a feature of other studies with the H1N1pdm vaccine, with and without adjuvant, in patients with AIRD [33–36]. In the current study, seroprotection after vaccination for all three seasonal antigens was reached in more than 85% patients for all medications used (Fig. 2), except for rituximab. Precisely, majority of rituximab-treated AIRD did not show an increase

Table 6 Long-term effects of vaccination with pandemic vaccine

H1N1pdm	Control	AIRD vaccinated once	AIRD vaccinated two times	Kruskal–Wallis + Dunn/Fischer exact test
Number (M/F)	3 (2 M/1F)	11 (3 M/8F)	29 (10 M/19F)	
Age (min–max)	41 (25–58)	63 (46–82)	53 (48–61)	
Samples taken before first vacc				
Time (days)—median (IQR)	0 (0–0)	0 (0–0)	0 (0–0)	
HI GMT titer (95% CI)	20 (3.5–111.9)	16.4 (4.2–63.8)	16.9 (9.8–29.2)	
Seroprotection	33% (1/3)	36% (4/11)	31% (9/29)	
Neg/borderline/pos	2/1/0	7/0/4	20/8/1	
Samples collected > 180 days after last vaccination				
Time (days)—median (IQR)	202 (194–205)	197 (183–210)	187 (182–192)	
HI GMT titer (95% CI)	45.8 (25.5–81.9)	40 (13.9–114.8)	43.6 (20.2–94.2)	
Seroprotection	100% (3/3)	82% (9/11)	45% (13/29)	AIRD1st vs AIRD 2nd *
Neg/borderline/pos	0/2/1	2/5/4	16/1/12	
Seroconversion	0/0	100% (1/1)	25% (2/8)	
Seroresponse	33% (1/3)	27% (3/11)	28% (8/29)	

Of AIRD vaccinated once only, one patient had baseline titer < 10, so seroconversion could be tracked, while in AIRD vaccinated twice, eight patients had baseline titer < 10

GMT geometric mean titer, *95% CI* 95% confidence interval, *IQR* interquartile range, *HI* hemagglutination inhibition

*$p < 0.05$ significance

in HI titers, while showing an increase in HI titer in more than 50% of patients treated with all other medications (methotrexate, adalimumab, etanercept, tocilizumab) (Fig. 3; Table 2). These data on patients, treated with rituximab, confirmed previous seasonal [26, 37, 38] and pandemic [24] influenza vaccination data. In addition, similar to the current study, other DMARDs [26, 39, 40] and glucocorticoids [41] do not decrease immunogenicity of influenza vaccine.

The degree of protection elicited by vaccination depends on the interplay between vaccine composition and circulating influenza viruses, the age of the vaccine recipient, and their previous exposure to influenza. Currently, inactivated vaccines are the most effective means to counteract influenza infection. They show over 60% ability to prevent morbidity and mortality in low-risk target populations, such as healthy adolescents or adults, but may have little effect in younger (naïve) or older (decreased immune function) populations, as well as over time, due to a low antigenic match. A HI titer of ≥ 40 is considered to be immunoprotective, but a titer over 80 is mostly considered as serological proof of natural infection [42]. Baseline HI GMT against all three antigens in seasonal vaccine was 40 and above, specifically for influenza H3N2 and B HI GMT was 80 or higher in vaccinated and non-vaccinated AIRD and controls, which is in line with findings of Jain et al. [43]. This could be due to the clinical and subclinical infections and vaccinations in previous influenza seasons. Due to the presence of high pre-existing HI titers of antibodies against influenza, we foresee benefit in the personalized medicine field in the future.

Additionally, we show for pandemic influenza that the second vaccination did not significantly change the GMT; it did not improve the seroprotection (Table 6) and did not lead to longer persistence of increased HI titers (Fig. 6) in those receiving also the second dose of the pandemic vaccine.

After receiving seasonal influenza vaccine, HI GMT significantly decreased at > 180 days in AIRD while not in controls (Table 4). As influenza vaccines are meant to provide protection for disease for entire season, our results show that the problem is not immunogenicity of vaccine in AIRD, but further scrutiny is needed for duration of vaccination effects in those patients. Due to low numbers of patients in each treatment group and differences among persistence of seroprotection to different antigens included in the vaccine, it is hard to draw conclusions, if persistence of seroresponse is affected by treatment (Fig. 4). We also confirmed that vaccination with pandemic vaccine did not improve persistence of seroresponse to antigens from seasonal influenza vaccine at > 180 days (Fig. 5).

In our analysis, we noticed that patients and controls vaccinated with seasonal influenza vaccines also had significantly increased titers of IgG and IgA antibodies against nucleoproteins of influenza A and B (ELISA A, B) after vaccination (Table 1). This was not seen after pandemic influenza immunization, with exception of level of IgA against influenza A which significantly increased after the first vaccination of AIRD patients (Table 5). Furthermore, in AIRD and controls whose HI titer to seasonal H1N1 changed > 4-fold after seasonal vaccination and had prior vaccination negative IgG and IgA nucleoprotein titers, positive nucleoprotein/matrix antibody titers were developed in 31–70%. In those whose HI titers did not change with vaccination only 0–20% also show the change in nucleoprotein/matrix IgG and IgA titers (Table 3). For the other two antigens, the difference was not so striking, but the trend was also observed (data not shown). In the pandemic vaccine, we did not observe this trend as those with HI change > 4-fold developed positive ELISA titers in 0–39% cases and if no HI change was observed development of positive ELISA was observed in 0–27% (Table 3). The seasonal influenza vaccine did not include adjuvants, while pandemic vaccine did. While in a certain percentage change from negative to positive nucloprotein/matrix IgG and IgA antibody titers and > 4-fold change in HI probably reflects natural infections with influenza, it is possible that such a high percentage (70%) is also due to a cross-reactivity with antigens included in seasonal influenza vaccine [44, 45].

The start of pandemic influenza virus season in Slovenia was in mid-June 2009, and it had two waves. The first wave had peak from July to August and second wave that lasted from beginning of November 2009 to the end of the year, with the peak of cases in week 47 (mid-November). Last cases of 2009/2010 season in Slovenia occurred in mid-February 2010. Our study started in mid-October and at that time a high proportion of general population was already infected with the pandemic H1N1pdm virus. Majority of infections were subclinical with mild respiratory symptoms. The late timing of influenza vaccination was already studied elsewhere, emphasizing the need of a timely reminder [46].

The overall percentage of seropositivity to the pandemic influenza virus among the Slovene population after second pandemic wave in study conducted on sera collected from February to September 2010 was high: 76.9% of the participants had antibody titers of ≥ 20. A titer of ≥ 40 was detected in 54% of the serum samples tested. There was no statistically significant difference in titers between individuals who had never been vaccinated with influenza vaccine and those vaccinated at least once. High seroprevalence to pandemic influenza has been found in all age groups regardless of the absence of clinical symptoms compatible with acute respiratory infection. Previous vaccinations with seasonal influenza vaccines had no impact on serological response to the influenza H1N1pdm 2009 virus. Influenza epidemic in the 2007/2008 season caused by the seasonal influenza H1N1 virus may have increased the prevalence of cross-reactive antibodies against predominant pandemic H1N1pdm 2009 virus [47] and also high H3N2 and B circulation in the previous season. High GMT and seroprotection against H1N1pdm at the inclusion

in our study, especially in vaccinated healthy controls, comprising mainly of health professional from Department of Rheumatology, National Laboratory for Health, Environment and Food and students of medicine is therefore not surprising.

We are aware of the limitations of this study that are very high baseline HI titers of antibodies to some vaccine antigens, low numbers of participants in our control group and absence of control group for second dose of pandemic vaccination. Our study started after the first wave of pandemic when many of participants could be already naturally infected with pandemic influenza H1N1pdm. However, our results are concordant with a few other studies [4] and support the compelling evidence on efficacy of inactivated purified surface fragments influenza vaccine in AIRD patients. As the safety of trivalent seasonal and monovalent pandemic influenza vaccines were already investigated in our previous report [9], with only transient changes in ANA titers (including the development of new ANA) and aCL IgG/IgM, we can now conclude that influenza vaccination is immunologically active for AIRD patients, with little value of the second dose of the pandemic vaccine and further scrutiny on persistence of immune response to vaccine in is AIRD needed.

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Compliance with ethical standards

Ethical approval for the study was obtained from the National Medical Ethics Committee of Slovenia (#122/09/09), and the study was conducted according to the principles outlined in the Declaration of Helsinki. All participants signed an informed consent.

Disclosures None.

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